# BINDING OF ADENINE NUCLEOTIDES TO MITOCHONDRIAL MEMBRANE

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## 1. Introduction

It has been reported from different laboratories [1–4] that mitochondria are capable of binding ATP. However the exact magnitude of binding, and the relevance of this process to the mechanism of oxidative phosphorylation are still unknown, due to the extremely low amounts of bound nucleotides, and to some difficulties in carrying out the measurements. The key to clarification of this problem has been the discovery in this laboratory [4] that $HClO_4$ treated mitochondria retain only a small portion of the originally bound ATP.

This paper describes a method for quenching mitochondrial reactions which, by substituting $HgCl_2$ for $HClO_4$ as the denaturating agent, easily permits to detect the binding of both ATP and ADP. The bound species can then be recovered, identified, and measured following acid extraction of the quenched mitochondrial pellet. Furthermore, the relationships between the binding of adenine nucleotides and the mechanism of oxidative phosphorylation are discussed. The results clearly show that the bound nucleotides are directly involved in the terminal step of phosphorylation of ADP.

## 2. Materials and methods

Mitochondria from rat livers were prepared by the procedure of Schneider [5], and from beef heart according to Blair [6]. Two different preparation of submitochondrial particles were employed, both derived from the 'ETPH ($Mg^{2+}$, $Mn^{2+}$)' particles described by Beyer [7]: in the type A particles 6 mM ATP was present during the sonication of mitochondria, in the type B particles ATP was omitted. Particles were used immediately after preparation. Protein was determined by a biuret method [8].

The procedure finally developed for the analysis of bound radioactive nucleotides was performed as follows: in the experiments with mitochondria the reaction was stopped by addition of 1 ml of 0.13 M $HgCl_2$ plus 0.1 ml of silicotungstic acid [9]; the mixture was then transferred in a centrifuge tube, the walls of the vessel washed with 2 ml of 6.5 mM $HgCl_2$, and the washing solution also transferred in the centrifuge tube. The complete mixture was centrifuged at 5°C for 30 sec at 15 000 *g* and the supernatant discarded, the walls of the tube wiped with filter paper, the pellet resuspended in 10 ml of 6.5 mM $HgCl_2$ and centrifuged as above. The washing procedure was then repeated once. Bound nucleotides were completely extracted from the mitochondrial pellet with 3 ml of cold 3% $HClO_4$ for 2 hr at 0°C. The acid extract was counted in a liquid scintillation spectrometer. The identification of bound species was carried out as follows: the $HClO_4$ extract was bubbled with $H_2S$ to remove excess $HgCl_2$. After centrifugation, the supernatant was neutralized, centrifuged, bubbled with air to remove excess $H_2S$, and analyzed enzymatically for adenine nucleotides [10]. Appropriate blanks were carried out as the assays tests except that the radioactive nucleotide was added 1 min after the addition of $HgCl_2$ plus silicotungstic acid mixture. The same procedure was followed for submitochondrial particles, except that

the reaction was stopped by addition of 1.4 ml of 0.2 $HgCl_2$ plus 30 $\mu$l of silicotungstic acid.

Phosphorylation was measured in the presence of hexokinase (EC 2.7.1.1), glucose and labelled ortophosphate [9]. The rate of ADP phosphorylation was linear for at least two min. ATP hydrolysis was determined as described before [11]. [$^{32}$P] $P_i$ was purified as described by Nielsen and Lehninger [12].

## 3. Results and discussion

### 3.1. *Binding of adenine nucleotides to rat liver mitochondria*

Complete data collected in table 1 show that mitochondria incubated with [$^{14}C_8$] ADP or [$^{14}C_8$] ATP retain a substantial amount of radioactivity after quenching with $HgCl_2$. The amount of ADP and ATP, as detected enzymatically, accounts for all the bound radioactivity. The table also shows that carboxyatractyloside (CAT) added prior to nucleotide strongly prevents the binding, although the overall amount of nucleotides, as measured enzymatically, is unaffected. When added after adenine nucleotide, CAT has no effect whatsoever on the binding of radioactivity. These findings establish unequivocally that the bound nucleotides are located inside the atractyloside barrier, and that CAT per se does not interfere with the binding process. Furthermore, the relative amounts of the nucleotides reflect the variations of the mitochondria ATP/ADP ratio: when CAT is added prior to radioactive nucleotide, thus preventing the exchange between the intra and extramitochondrial compartments, ATP is prevalently bound, reflecting the phosphorylation of endogenous ADP.

The experiments reported in table 2 give some insight on the significance of bound nucleotides. In A) mitochondria were incubated for 5 sec in the presence of respiratory substrate (+ phosphate). Previous experiments have shown that at this early stage only a part of endogenous ADP is phosphorylated. Now, if a small amount (12 nmol) of highly radioactive ADP is added, and the reaction stopped after about 0.5 sec, the specific activity of bound ATP is much higher than that of the free species. It is therefore evident that the first ATP being formed upon ADP phosphorylation is in reality bound ATP. In B) is reported the reverse experiment, that is: mitochondria were preincubated for 5 min in order to get high intramitochondrial ATP level; the uncoupler and a small amount (12 nmol) of radioactive ATP was then added and the reaction stopped after about 0.5 sec. As shows in the table, the specific activity of bound ADP was higher than that of free ADP, suggesting that bound ADP is the first ADP formed upon ATP hydrolysis. These findings therefore indicate that at least part of the bound ATP and ADP are directly connected to oxidative phosphorylation.

Table 1
Bound adenine nucleotides to rat liver mitochondria

| Sequence of additions (in brackets incub. time) | Radioactivity | Bound adenine nucleotides (nmol·mg$^{-1}$) ADP | ATP | ADP+ATP (enzymatic analysis) |
|---|---|---|---|---|
| [$^{14}$C]ADP (4′) | 3.96 | 2.86 | 1.04 | 3.90 |
| [$^{14}$C]ATP (4′) | 4.05 | 2.00 | 1.15 | 4.15 |
| CAT. (I′) [$^{14}$C]ADP (4′) | 1.08 | 0.95 | 2.89 | 3.84 |
| CAT. (I′) [$^{14}$C]ATP (4′) | 1.46 | 1.03 | 2.78 | 3.81 |
| [$^{14}$C]ADP (4′) CAT. (I′) | 3.90 | 2.48 | 1.43 | 3.91 |
| [$^{14}$C]ATP (4′) CAT. (I′) | 4.35 | 1.27 | 2.70 | 3.97 |

Incubation system contained in a vol of 2 ml: 90 mM sucrose, 50 mM KCl, 50 mM Tris–HCl (pH 7.4), 5 mM glutamate, 1 mM [$^{14}C_8$]ADP or 1 mM [$^{14}C_8$]ATP (150 cpm/nmol) and 30 mg of mitochondrial protein. Temp. 18°C. Where added carboxyatractyloside (CAT·) was 250 $\mu$M.

Table 2
Bound and free ATP synthesized by oxidative phosphorylation; bound and free ADP formed during uncoupler induced ATPase

| Event | Sequence of additions (incubation time in brackets) | cpm/nmol ATP Bound | ATP Free | ADP Bound | ADP Free |
|---|---|---|---|---|---|
| A) ATP synthesis | Mito. (5″) [$^{14}$C]ADP (0.5″) | 426 | 250 | – | – |
| B) ATP hydrolysis | Mito. (5′) DNP + [$^{14}$C]ATP (0.5″) | – | – | 256 | 160 |

Incubation systems as described in table 1. Phosphate (2 mM) and EDTA (2 mM) only in experiment A) were added. After the reaction was stopped with $HgCl_2$ + silico-tungstic acid, the mixture was centrifuged, the supernatant discarded (it does not contain appreciable amounts of adenine nucleotides) and both the walls of the centrifuge tube and the surface of the pellets were carefully washed with a mixture containing: 0.13 M $HgCl_2$ (1 ml), silicotungstic acid (0.1 ml) and incubation medium (1.8 ml). After draining, the tubes were wiped with filter paper and the pellet suspended in 5 ml of 6.5 mM $HgCl_2$. After centrifugation the supernatant was acidified with $HClO_4$. The pellet was extracted at 0°C with 3 ml of cold 3% $HClO_4$. From the supernatant (which contains free ADP and ATP) and the acids extracts (which contain bound ADP and ATP) excess $HgCl_2$ and $H_2S$ were removed as described under Methods. Aliquots were analyzed for ADP and ATP enzymatically [10], aliquots were separated by chromatography [17] and counted.

### 3.2. *Binding of ATP to submitochondrial particles and ATPase activity*

In the experiments with intact mitochondria the actual intramitochondrial ATP/ADP ratio is modulated by highly complicated permeability control. This is not the case of sonicated vesicles, where the analysis of ATP and ADP binding can be simply carried out as a function of the adenine nucleotides concentration. As it can be seen in fig.1, the binding of ATP-Mg (open circles) elicits a three-stepped saturation curve up to a maximum of 1.5–1.8 nmol per mg protein. In a long series of experiments, the contribution of each saturation curve to the total binding capacity was approximately the same.

If this sort of binding represents the preliminary and necessary event for the hydrolysis of ATP, one would expect a similar pattern for the kinetics of ATPase activity. This was indeed the case, and the results are also reported in fig.1 (closed circles). It is to be recalled that the experiments of ATP binding were carried out with type A particles, while the ATPase was measured in type B particles. This latter was mandatory due to the very poor ATPase activity of type A particles, in contrast with type B particles which have high uncoupler induced ATPase, presumably due to the leakage of $F_1$ inhibitor [13]. We must stress at this point, on the other hand, that, as far as the ATP binding is concerned, no difference could be detected between the two types of vesicles. Finally, it is unlikely that the three distinct

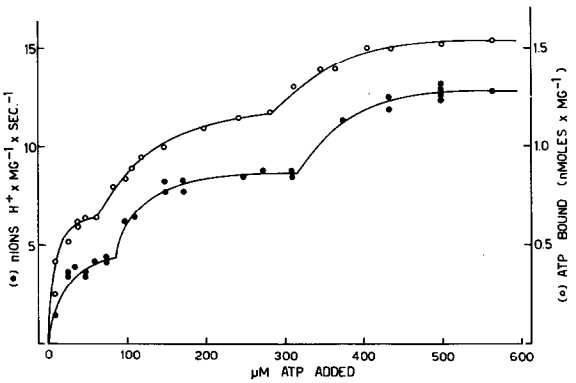


Fig.1. Binding of [$^{14}$C]ATP to submitochondrial particles and ATPase activity. A) The binding of [$^{14}$C]ATP-$Mg^{2+}$ (○) was detected by incubating 7 mg particles in a medium containing in 2.2 ml: 250 mM sucrose, 10 mM inorganic phosphate (pH 7.0), 7.5 mM NADH, 6 μg oligomycin. Temperature 20°C. After 2 min preincubation the reaction was initiated by addition of [$^{14}C_8$]ATP-$Mg^{2+}$ (300 cpm/nmol), allowed to proceed for 20 sec and stopped by $HgCl_2$ plus silicotungstic acid (see Methods). B) ATPase activity (•) was determined (see under Methods) in a medium containing in 2 ml: 250 mM sucrose, 5 mM Tris–MES (pH 6.9) 0.7 μM FCCP. Particles (0.112 mg protein) were preincubated for 2 min at 20°C before addition of ATP-$Mg^{2+}$.

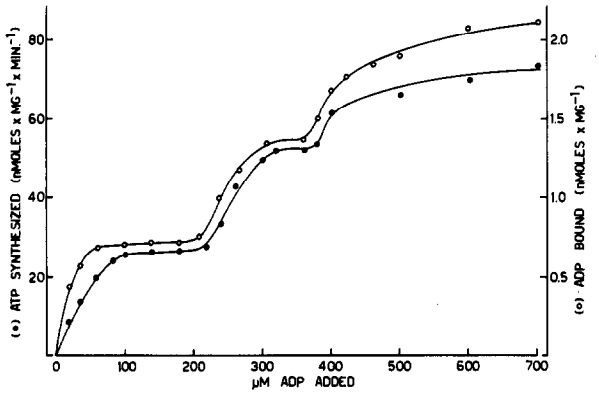


Fig.2. Binding of [$^{14}$C]ADP to submitochondrial particles and ADP phosphorylation. A) The binding of [$^{14}C_6$]ADP-$Mg^{2+}$ was tested as described in fig.1. [$^{14}C_6$]ADP-$Mg^{2+}$ (300 cpm/nmol) was used. B) ADP phosphorylation was determined by incubating particles (3 mg) in a medium containing in 2 ml: 250 mM sucrose, 10 mM [$^{32}$P]$P_i$ (pH 7.2), 2.5 mM NADH, 15 mM glucose, 0.5 mM $MgCl_2$, 20 EU hexokinase (EC 2.7.1.1) and ADP-$Mg^{2+}$ at the concentrations shown in the figure.

saturation curves of ATP binding are the result of different particles populations, because the same results were obtained in particles prepared with different procedures.

### 3.3. *Binding of ADP to submitochondrial particles and ADP phosphorylation*

Fig.2 (open circles) illustrates the binding of ADP as a function of ADP-Mg concentration: here again the binding shows three distinct saturation curves, which equally contribute to the overall binding capacity (about 2 nmol per mg protein). This kinetics mimics the kinetics of ADP phosphorylation, which shows three different velocities, each one of them equally contributing to the overall phosphorylation*. Essentially identical results were obtained with type A and type B particles. The peculiar kinetics of nucleotide binding, ATPase and of ADP phosphorylation could indicate a possible co-operativity of three distinct ATP forming systems. Evidence for this possibility comes from the observation that in mitochondria the block of electron flow at the level of each energy transducing site lowers the rate of ATPase by one third of the overall rate [11]. Furthermore, the utilization of the respiratory energy for the movements of calcium is also mediated by three operationally distinct transducing devices [16].

* Data from different laboratories give values for the $K_m$ (ADP) for oxidative phosphorylation in sonicated particles ranging from 12 μM [14] to over 300 μM [15]. These discrepancies probably reflect the result of different preparation procedures. Experiments on this point are in progress in this laboratory.

## 4. Conclusions

The results presented can be summarized as follows: a) intact mitochondria and submitochondrial vesicles bind nucleotides in a compartment which is located inside the atractyloside barrier. b) In mitochondria the phosphorylation of ADP leads primarily to the formation of bound ATP; conversely, dephosphorylation of intramitochondrial ATP leads primarily to the bound ADP. c) In submitochondrial particles both ADP and ATP binding elicit three saturation curves which mime the kinetics of the uncoupler stimulated ATPase and of ADP phosphorylation.

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